Polarization Dependence of the Raman Scattering of Oriented Porcine Muscle Fibers Affected by Storage Time and Spoilage

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Abstract

Polarized Raman spectroscopy is shown to be a suitable tool to obtain more information about meat spoilage mechanism. Furthermore, it is well suited to monitor the anisotropic properties of oriented muscle fibers during storage. Fresh porcine musculus longissimus dorsi was used as the test sample**.** Polarized Raman measurements were performed for a period of 14 days. A microsystem diode laser with an excitation wavelength of 671 nm as light source was utilized. The excitation laser beam was polarized perpendicular and parallel to the long axis of the muscle fibers. By means of Raman spectra and principal components analysis, a distinction according to the two muscle fiber orientations (parallel and perpendicular) was observed for untreated and sterilized meat. Due to the increase of fluorescence background caused by the microbial spoilage of the meat surface, a decrease of the anisotropy of the untreated meat was found for the storage periods in Raman spectra. The high fluorescence background obscured the polarization information detected on the meat surface. As for the sterilized samples with very low bacterial load the distinction between the two orientations remained pronounced until the last day of storage. So this obscurity of the polarization information in Raman spectra can be a new indicator for meat spoilage.

Keywords

Polarized Raman Spectra; Oriented Muscle Fibers; Microbial Reference Analysis; Difference Spectra; PCA; Storage-time Dependence

Introduction

Raman spectroscopy is a viable tool for the rapid detection of meat spoilage (Schmidt et al., 2009; Sowoidnich et al., 2010). While it is shown that the microbial activity produces an increasing fluorescence background contributing to the detection of spoilage, the role of muscle fiber changes during storage is a matter of debate (Sowoidnich et al., 2012). To gain deeper insight into the mechanism of the Raman detection of meat spoilage, a study of the change of the fiber properties is required. Muscle fibers, which are characterized by a high organization of the contractile proteins in myofibrils, are a major building block of meat. These, myofibrils are composed of repeating sections of sarcomeres (Craig & Padron, 2004; Lawrie, 1998). Additionally, myofibrils are strongly birefringent due to their A (anisotropic) and I (isotropic) bands, and this enables them to be investigated with polarized light (Kerry et al., 2002). Moreover, the α-helical segments of the fibrous protein assemblies are highly oriented along the fiber axis which causes optical anisotropy (Pezolet et al., 1980).

By applying polarized light, data about the muscle fiber orientation and order can be obtained.

Hence, polarized photometric light was used to measure the optical transmittance of oriented red and white muscle fibers of pork at selected days of storage (Swatland, 2003). Additionally, polarized front face fluorescence was used to assess the angular anisotropic distribution of the detected intensities of oriented muscle fibers (Luc et al., 2008). However, fluorescence spectroscopy is biased by thermal variations requiring careful control of the temperature (Demchenko & Ladokhin, 1988).

As a primary study for the possibility to use near infrared (NIR) spectroscopy in meat tenderness investigation, measurements were performed on pork and beef muscles (Swatland, 1996). It was found that

using polarized light on stretched sarcomeres increased the detectable levels of backscattered NIR. However, a major drawback of infrared spectroscopy is the high absorption of water (Blout & Lenorman, 1953). Hence, due to the fact that fresh meat consists of roughly 75 % water, this technique is inappropriate to measure meat muscle fibers (Fjelkner-Modig & Tornberg, 1986). Unlike IR spectroscopy, Raman spectroscopy exhibits only a weak signal of water in the spectral range from 700 to 1800 cm^{-1} at around 1650 cm^{-1} which is not disturbing. Furthermore, the Raman spectra provide specific fingerprints of the molecular structure and the biochemical compositions of meat.

By means of Raman spectroscopy, the secondary structure of the polypeptide's backbone of proteins can be characterized (Carew et al., 1975; Lippert et al., 1976). The sensitivity of polarized Raman spectroscopy has been proved on biological tissues for the investigating of structured materials (Smith & Berger, 2005). The polarized Raman spectra of glycerinated and intact muscle fibers of the giant barnacle showed that the conformation-sensitive amide I, amide III and C-C stretching modes yielded higher Raman intensities when the polarization of excitation laser and the scattered light were parallel to the muscle fiber axis compared to perpendicular orientation (Pezolet et al., 1988).

In our investigation, an ensemble of pork muscle fibers is studied on intact samples of skeletal muscle tissue undergoing an aging and spoilage processes. The experiments are performed directly on the muscles with respect to in-situ investigations. This paper presents polarized Raman scattering as a potential tool for evaluating the anisotropic properties of muscular structures during storage time.

For this purpose, the anisotropic changes in Raman spectra of untreated and sterilized pork meat of two orientations of the muscle fibers (parallel and perpendicular) are studied in the presence and absence of bacterial spoilage for 14 days of storage after slaughtering. To our knowledge, this is the first investigation of using polarized Raman spectroscopy of oriented muscle fibers on mammalian meat correlated with time-dependent storage.

To monitor presence or absence of microbial spoilage, the total number of viable aerobic mesophilic plate counts is determined for the untreated and the sterilized samples (Borch & Kant-Muermans, 1996). Principal components analysis (PCA) is used as a statistical method to evaluate the structural properties of pork meat during storage time.

Experimental Section

Sample Preparation

As test sample, *musculus longissimus dorsi* (LD) was chosen for our investigation due to its homogeneity. Six entire muscles from three pork carcasses (Bayerisch-Hybrid-DL) were procured from a local abattoir 24 hours p.m. The left muscles were used as obtained for the spoilage experiment, while the right muscles were sterilized in a 5% sodium hypochlorite solution to eliminate surface contamination (Sowoidnich et al., 2012) and handled sterile in a control experiment. The muscles were cut along the fiber orientation into 14 slices with a thickness of approximately 1.5 cm. Each slice was halved and packed separately in Petri dishes which were provided with a sterile filter paper wetted with 1 ml saline to avoid desiccation during storage.

Half of the slices was transported chilled to Technical University Berlin for the Raman investigations, whereas the other half was used for microbial reference analysis at University Bayreuth, Research Center of Food Quality concurrently to Raman measurement. All slices were stored at 5 °C in a laboratory refrigerator (Spezial-468, Philipp Kirsch, Germany) for 14 days.

One slice of each muscle was used per day. For the Raman measurements two samples were cut out vertically to the fiber axis using a cylindrical knife and respecting the arrangement of the fiber direction in the meat slice. Then the subsamples were mounted in PVC tubes and put in Petri dishes to prevent them from dehydration.

Raman Measurements

Fig.1 shows the experimental setup. A microsystem diode laser supplied by Ferdinand-Braun-Institut, Leibniz-Institut für Höchstfrequenztechnik and operating at 671 nm (Maiwald et al., 2008) was applied as excited light source. The laser power at the sample was 50 mW with integration times of 1 to 5 s for one Raman spectrum. The laser beam was passing through a band pass filter (2) (Semrock, Inc.), and polarized by a Glan-Thompson linear polarizer (3) (Bernhard Halle Nachfl. GmbH). Then, two dielectric mirrors (4), (5) (ThorLabs GmbH) guided the polarized beam towards a Raman edge filter (9) (LOT Oriel Group) which

reflected the beam to a lens (6) with a focal length of 30 mm. By this lens, the laser beam was focused through a quartz window (7) (ThorLabs GmbH) which avoided dehydration of the sample during measurement and which kept the muscle fiber alignment fixed. The meat sample was mounted in a scaled rotatable holder (8) to adjust the muscle fiber orientation. The lens (6) also served to collect the back scattered radiation from the sample. The set of two Raman edge filters (9) blocked the Rayleigh scattered radiation and the anti-Stokes signals. Only the Stokes-shifted signals passed through a telescope consisting of lenses (10) and (11) with f = 50 mm and f = 25 mm, respectively. (all lenses were from ThorLabs GmbH). The collected Raman signals were polarized parallel to the polarization direction of the excitation radiation with a second Glan-Thompson polarizer (see above) (12). By means of the lens (13) (with focal length of 50 mm) the polarized Raman signals were focused on the entrance slit of the spectrograph (14) (Chromex 250IS). A charge coupled device (15) (EHRB 1340 x 400, Princeton Instruments) operating at -70 °C was used to detect the spectra. A computer (16) was used to process the Raman spectra running Winspec software (Roper Scientific).

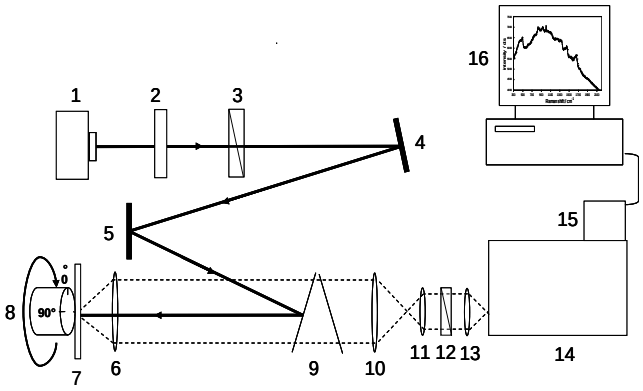

FIG. 1 EXPERIMENTAL SETUP; (1) 671 NM MICROSYSTEM DIODE LASER, (2) BAND PASS FILTER, (3) (12) POLARIZERS, (4)(5) DIELECTRIC MIRRORS, (6) (10)(11)(13) LENSES, (7) QUARTZ WINDOW, (8) MEAT SAMPLE IN SCALED ROTATABLE HOLDER, (9) RAMAN EDGE FILTERS, (14) SPECTROGRAPH, (15) CCD, AND (16) COMPUTER

The polarized Raman spectra were recorded at two directions of the muscle fiber axis, either parallel or perpendicular to the polarized incident laser beam. At each angular position, 10 Raman spectra were detected for 5 different spots of the sample, where the sample holder was rotated in increments of 90° from 0° up to 270°. This means that in total 100 Raman spectra were recorded at each fiber orientation per muscle at each day of storage.

Due to an increase of fluorescence background in the untreated LD, the integration times were reduced from 5 s for fresh meat to 1 s for spoiled meat. For the sterilized LD the integration times were 5 s for all investigated days.

Data Processing

PCA (Beebe et al., 1998; Miller, 2005; Brereton, 2007) was utilized to analyze the recorded Raman spectra by means of MatLab (MathWorks Inc. Natick, MA) combined with PLS-Toolbox (Eigenvector Research Inc., Wenatchee, WA). The data preprocessing was performed using mean centering and Savitzky-Golay smoothing with second derivative computation.

Microbiological Analysis

In parallel to the Raman measurements, microbiological analyses were performed to monitor the progress of meat spoilage. To this end, the total number of viable plate counts of mesophilic, aerobic microorganisms on the meat surface was determined at University Bayreuth, Research Center of Food Quality. For each muscle and day, a cylindrical sample with 5 cm^2 area was cut out and homogenized with 20 ml of saline in a Stomacher bag. The homogenized samples were serially diluted using 1 ml sample and 9 ml saline for each sample, from which 100 µl aliquots at three appropriate dilution levels were plated in triplicate on Tryptic Soy Agar (TSA) plates (Oxoid) and incubated at 30 °C for 48 h. For the sterilized meat as control, the undiluted homogenised samples were plated in triplicate. Then, the number of colony-forming units (cfu) was counted.

Results and Discussion

Microbiological Determination of Bacterial Spoilage

The untreated meat samples show bacterial spoilage during storage at 5 °C. Fig.2 displays the bacterial growth kinetics averaged for the untreated three left porcine muscles LD stored in Petri dishes for 14 days at 5 °C.

The first two days represent the initial lag phase of the bacteria on the meat surface with surface loads in the range of 10^2 cfu/cm^2. A biphasic exponential growth of bacteria during storage is observed on the meat surface from the 3rd day until the last day of storage. The surface load of 10^6 cfu/cm^2 is reached between the 6th day and the 7th day. This threshold is the relevant limit in the production process of meat. At the 7th day to the 8th day the growth stagnates and enters a second

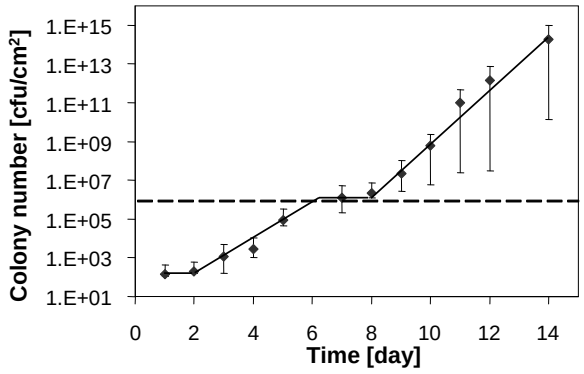

FIG. 2 EXPERIMENTALLY DETERMINED NUMBER OF COLONY-FORMING UNITS (CFU) ON THE MEAT SURFACE OF UNTREATED PORK LD, STORED AT 5 °C. THE DASHED LINE INDICATES THE 10^6 CFU/CM² THRESHOLD VALUE. ERROR BARS DENOTE THE MIN/MAX VALUES OF THE THREE MUSCLES

phase with continuous growth to reach a surface load of around 10^{15} cfu/cm² after 14 days. The error bars display the maximum and the minimum values of colony-forming units detected on the three muscles. They indicate that after 8 days the microorganisms develop in a different way on the three muscles. In contrast, the microbiological analyses of sterilized muscles shows that the concentration of bacteria on the meat surface remains always below the detection limit of 200 cfu/cm².

Polarized Raman Spectroscopy

The intensity of the fluorescence background of the Raman spectra is in accordance with previous observations (Jordan et al., 2009), i.e. low fluorescence until the 6th day, a slow increase until the 9th day and a very strong increase of the background fluorescence after the 10th day of storage.

To remove the fluorescence background, a 5th order polynomial fit with 6 reference points is subtracted as shown in Fig. 3. The unprocessed Raman spectrum of LD is curve (a), the dashed line refers to the baseline curve and the corrected spectrum is curve (b). Now, the weaker Raman signals can be better recognized, such as those of the protein backbone (υCN, υCC) at (1110 cm^{-1}, 1090 cm^{-1}), and tryptophan (Trp) at 1555 cm^{-1}.

In Fig. 4, storage-time dependent baseline-corrected Raman spectra of the untreated LD meat are shown for parallel (a) and perpendicular (b) orientation of the muscle fibers. For clarity the spectra are normalized to the net intensity of the Phenylalanine (Phe) peak at 1005 cm^{-1} and only 4 averaged spectra of selected days are displayed. For comparison, the corresponding spectra of the sterilized meat are displayed in Fig. 5. The typical Raman bands of amide I at 1650 cm^{-1}, amide III at 1312 cm^{-1}, and C-C stretch at 940 cm^{-1}, 900 cm^{-1} for α-helical proteins (Pezolet et al., 1978) can be observed for the two orientations of untreated and sterilized meat. In addition, to the strong CH bending modes at 1450 cm^{-1}, vibrations attributed to the aromatic amino acids Phe at 1005 cm^{-1}, Tyrosine (Tyr) at (858 cm^{-1}, 829 cm^{-1}), and tryptophan (Trp) at 1555 cm^{-1} as well as characteristic bands of residues and of the protein backbone (υCN, υCC) at (1110 cm^{-1}, 1090 cm^{-1}), and υCN at 1128 cm^{-1} are clearly identified (Careche et al., 1999).

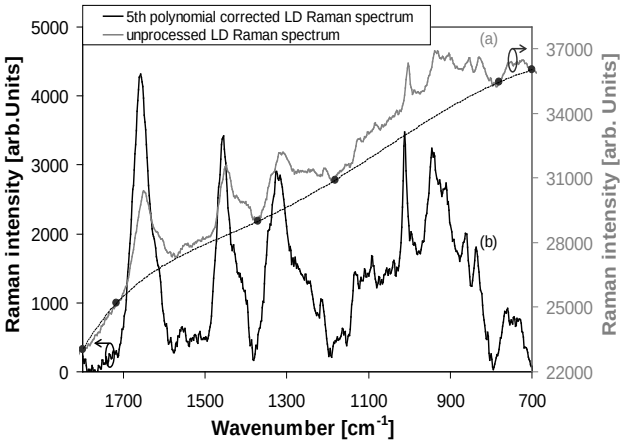

FIG. 3 RAMAN SPECTRA OF LD: (A) UNPROCESSED RAMAN SPECTRUM, (B) BACKGROUND-CORRECTED SPECTRUM USING 5TH ORDER POLYNOMIAL FIT

The two figures also show that both, the untreated and the sterilized LD corrected Raman spectra display more intense signals of helical proteins in the parallel state compared to the spectra of the perpendicular orientation. The difference spectra between both orientations (parallel-perpendicular) display the polarization-dependent information about orientation and storage effects on the untreated samples Fig.4 (c), as well as for the sterilized samples Fig.5 (c).

It is clearly recognized in the difference spectra of the untreated and the sterilized LD that the bands of amide I, III, and C-C stretch indicate high degree of oriented α-helical segments (Pezolet et al., 1978). The difference spectra also reveal polarization and orientation dependent signals of υ(=C-C=) stretching vibration of conjugated polyene chains at 1160 cm^{-1} (Pezolet et al., 1978) and CH_2 bending vibration contributed with some Trp lines at 1450 cm^{-1}, which is in accordance with (Pezolet et al., 1988) showing that

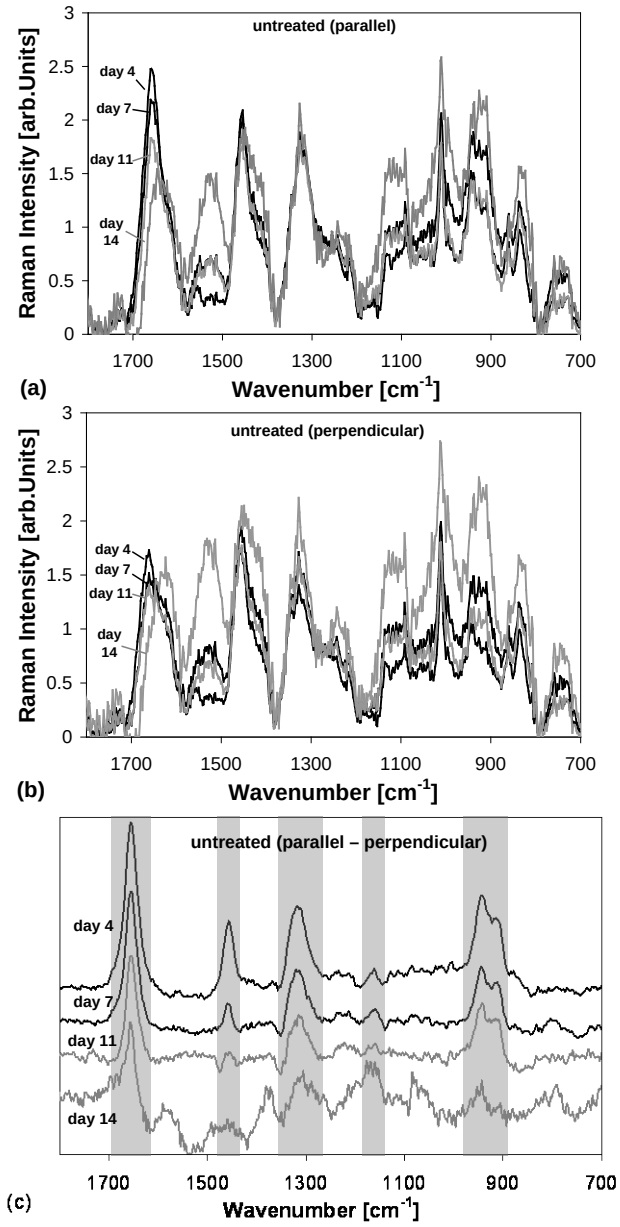

FIG. 4 BACKGROUND CORRECTED AND NORMALIZED RAMAN SPECTRA OF UNTREATED PORCINE LD WITH MUSCLE FIBER ORIENTATION PARALLEL (A) AND PERPENDICULAR (B) TO THE POLARIZATION OF THE LASER BEAM FOR SELECTED DAYS OF STORAGE, AND THE DIFFERENCE SPECTRA (C) OF (A) MINUS (B), GRAY BARS INDICATE POLARIZED RAMAN BANDS

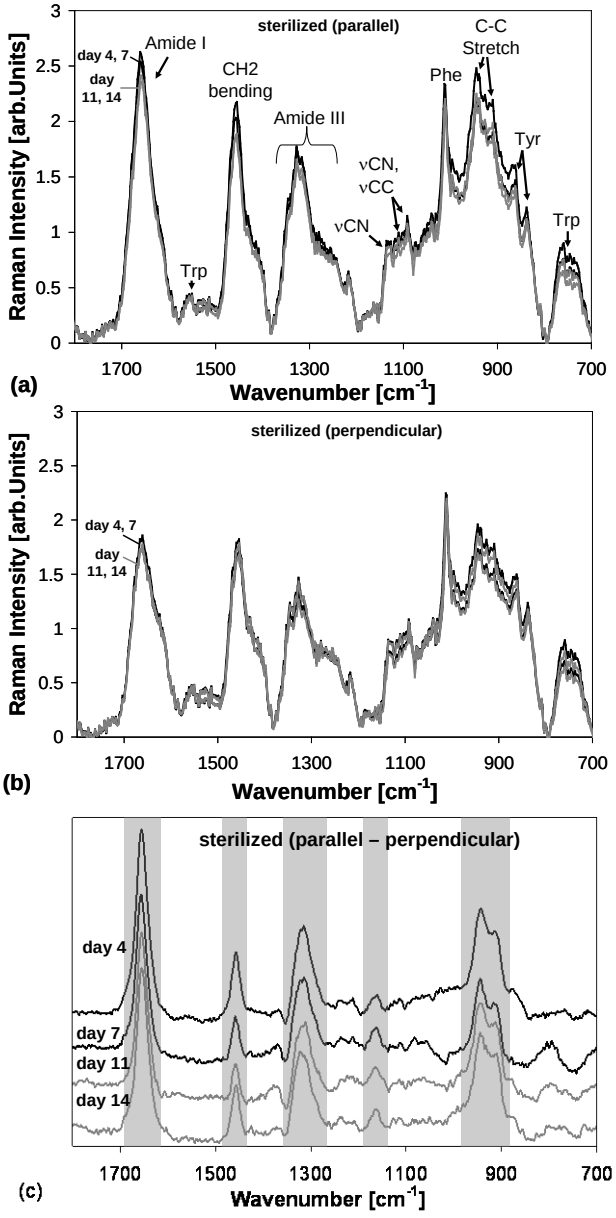

FIG. 5 BACKGROUND CORRECTED AND NORMALIZED RAMAN SPECTRA OF STERILIZED PORCINE LD WITH MUSCLE FIBER ORIENTATION PARALLEL (A) AND PERPENDICULAR (B) TO THE POLARIZATION OF THE LASER BEAM FOR SELECTED DAYS OF STORAGE, AND THE DIFFERENCE SPECTRA (C) OF (A) MINUS (B), GRAY BARS INDICATE POLARIZED RAMAN BANDS

the 1450 cm^{-1} band is unsuitable for intensity normalization of the spectra. For the untreated meat, not only a decrease of the net intensity of the Raman bands is observed for both orientations during storage, the differences between both fiber orientations are also decreasing when the meat is spoiled. Furthermore, the high fluorescence background at the last day of storage of untreated samples disturbs the polarization information detected of the oriented muscular structures on meat surface, as shown in Fig.4 (c).

For the sterilized meat, no apparent reduction in the net intensities during storage is observed. Furthermore, the difference spectra in Fig. 5 (c) show that the polarization-dependent distinction of both fiber orientations was maintained for 14 days of storage.

To probe the anisotropic properties for the two orientations (parallel and perpendicular) of the untreated and the sterilized LD muscles depending on storage time, PCA as a multivariate statistical method is applied on Raman spectra with the spectral region

from 700 to 1800 cm^{-1} for all days of storage. Briefly, PCA is used to identify spectral patterns representing variations in the dataset which are attributed to principal components (PCs). The first principal component (PC 1) accounts for the largest variability in the data, and each succeeding PC accounts for the remaining variability in decreasing order (Beebe et al., 1998; Miller, 2005; Brereton, 2007). Using these patterns, the spectra can be scored for each PC by calculating the scalar product between spectrum and loading. In that way, each spectrum is reduced to a single value for each PC.

For reasons of clarity and to simplify the score plots, averaged unprocessed Raman spectra are used as input data. To this end, 30 Raman spectra are averaged from 3 meat slices for the three animals, for each orientation, for untreated and sterilized samples and for each measuring day.

Fig.6 displays the corresponding scores plot of the untreated and the sterilized meat spectra for the first and the second principal components which explain 69 % and 21 % of the total variance in the dataset. Two distinct separations can be observed for both the untreated and the sterilized samples. PC 1 describes the relative intensity with which the averaged meat Raman spectrum is observed. For the untreated samples, this refers to the time-dependent changes of the Raman spectra, as the signal-to-background intensity ratio of the meat Raman pattern is decreasing with spoilage (see Fig.4 (a)). Both orientations (parallel and perpendicular) have a similar distribution along

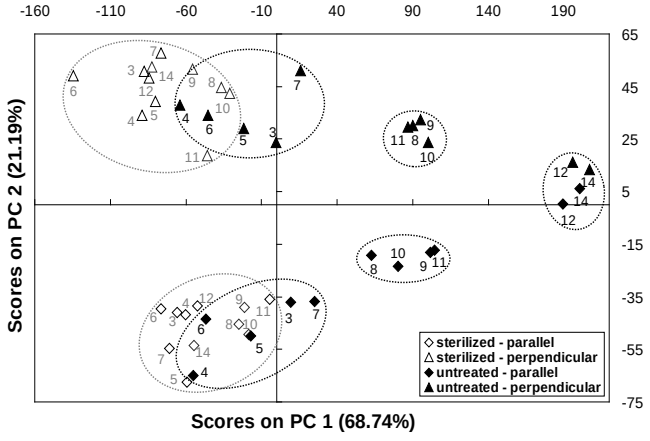

FIG. 6 RAMAN SPECTRA SCORED FOR PC 1 AND PC 2 FOR UNTREATED AND STERILIZED LD MUSCLE FIBERS FOR TWO DIRECTIONS (PARALLEL AND PERPENDICULAR) AND 14 DAYS OF STORAGE. PC 1 DISPLAYS TIME-DEPENDENT CHANGES, WHILE PC 2 DISPLAYS THE DECREASE OF ANISOTROPIC PROPERTIES OF THE UNTREATED MUSCLE FIBERS DURING STORAGE

PC 1. Three separated groups can be identified for the untreated meat, in the scores plot. This is in accordance with (Sowoidnich et al., 2012). One group includes the samples of the first 7 days of storage as edible meat.

The second group containing the 8^{th} day to the 11^{th} day of storage can be associated with a period of advanced spoilage of the meat while the third group comprises the heavily spoiled meat.

This age-dependent classification of the untreated samples is related to the bacterial growth kinetics on the meat surface (see Fig.2) where the first 7 days are characterized by bacterial surface loads below the threshold value of 10^6 cfu/cm^2.

A continuous increase of the bacterial surface load (cfu) beyond this threshold is typical for the second period of storage which is reaching a surface load up to 10^{11} cfu/cm^2. The third period reached up to 10^{15} cfu/cm^2. The scores distribution of the PCA shows that the first days as fresh samples are almost located in the negative part in the PCA. Then, due to aging and spoilage processes, the second group which includes spoiled samples is clustered in the positive part, whereas the last group of heavily spoiled meat is located in the furthest side of the positive part according to PC 1.

In contrast to the untreated meat, according to PC 1 no significant separation can be found for the sterilized meat. All sterilized samples are grouped in the negative part of PC 1. This is in agreement with the microbiological analyses showing that the concentration of bacteria on the meat surface remains below the detection limit of 200 cfu/cm^2.

Additionally, in the scores plot in Fig.6, PC 2 discriminates the two orientations of the muscle fibers with negative values for parallel state and positive values for perpendicular state for both, the untreated and the sterilized meat samples. The first 7 days of the untreated meat exhibit a clear separation between the two orientations. Then, with storage time and increasing spoilage, this difference decreases and vanishes for the heavily spoiled samples. This can be understood as a decrease of the anisotropy of the Raman spectra between the two fiber orientations. We can explain this decrease due to the presence of bacteria on the meat surface forming a film in the final phase (Schmidt et al., 2009). The bacteria arrange randomly on the meat surface, this leads to an isotropic detection of the recorded bacterial signals

masking the Raman signals of the meat. Furthermore, multiple scattering by the bacteria reduces the polarization information of the scattered photons which are sent back from the measured meat sample.

Accordingly, for the sterilized meat only a minor decrease in the anisotropy of the Raman spectra can be observed during storage time. The distinction between the two orientations of the muscle fibers is pronounced for all examined days. This means that the muscle fibers keep their anisotropic properties during storage and that the loss of anisotropy in the spectra of spoiled meat is related to the bacteria on the surface.

The first two loadings of the PCA are shown in Fig.7 in the spectral range from 700 to 1800 cm^{-1}.

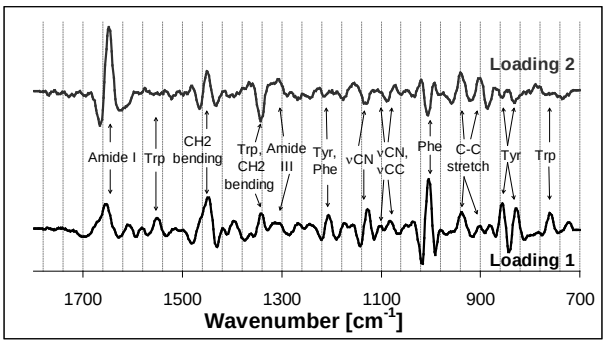

FIG. 7 LOADING 1 AND 2 FROM PCA OF ORIENTED UNTREATED AND STERILIZED LD MUSCLE FIBERS REPRESENTING SPOILAGE AND ORIENTATION EFFECTS, RESPECTIVELY

Loading 1 displays the typical protein structure of meat which is affected by aging and spoilage effects during time-dependent storage (Schmidt et al., 2010). Loading 2 represents the Raman bands which are sensitive to the orientation of the muscle fibers relative to the polarization of the light (Pezolet et al., 1988) revealing variation for the amide I band at 1650 cm^{-1}, CH_2 bending at 1450 cm^{-1}, as well as small changes for the amide III band at 1312 cm^{-1}, and C-C stretching of the protein backbone at 940 $cm^{-1}$and 900 cm^{-1}. These bands are all related to highly ordered α-helical domains. Furthermore, bands from Trp and C-H bending at 1340 cm^{-1} and Phe at 1005 cm^{-1} can be observed. When comparing both loadings of Fig. 7 with those published for packaged meat (Schmidt et al., 2010) and unpackaged meat (Sowoidnich et al., 2012) the similarities are striking. For the first loading representing the meat spectrum this was to be expected, but not for the second loading as this is representing the difference between the two fiber orientations which was not controlled in the earlier publications.

Conclusions

Polarized Raman measurements were performed for parallel and perpendicular orientation of muscle fibers of untreated and sterilized pork meat by means of a 671 nm microsystem diode laser for 14 days of storage. The spectra allowed for a clear discrimination of the fiber orientation as well as for the detection of spoiled and unspoiled samples as was shown with PCA by using the first two principal components. The distinction of the fiber orientation was related to polarization sensitive signals which can be assigned mainly to the highly ordered α-helical protein domains in the myofibrils (notably amide I, C-H-bending, amide III and two C-C skeletal stretching modes). The anisotropy of the muscle fibers was indicated by loading 2. When the untreated meat samples spoiled, this anisotropy of the spectra was damped and vanished when the meat surface was completely covered by bacteria. As the spectra of the sterilized meat samples maintained their distinct separation for both fiber orientations, we conclude that the bacterial growth on the meat surface can be considered as the main causative agent of reducing the anisotropic properties of the Raman spectra during storage time. The bacterial layer on the meat surface obscured the original Raman scattering of the underlying muscle fibers physically or by means of multiple scattering.

Due to the similarity of the second loading which represented the anisotropy of the meat fibers with the second loading of the PCA which was used in earlier work (Schmidt et al., 2010; Sowoidnich et al., 2012) for the discrimination of spoilage we may conclude that this part of the detection of spoilage was based on the loss of polarization information from the spectra due to the presence of bacteria.

In conclusions, this polarized Raman study on spoiled and sterilized meat has contributed to further understanding the mechanism of the Raman detection of meat spoilage showing that in addition to the fluorescence generated by bacteria on the meat surface, the bacteria also damp polarization–dependent Raman signals of α-helical protein domains in the muscle fibers which are the major cause for the optical anisotropy of muscle fibers.

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